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Epigenetic and genetic analysis of p16 in dermal fibroblasts from type 1 diabetic patients with nephropathy

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Background. Several studies have shown that cultured skin fibroblasts from patients with diabetic nephropathy (DN) exhibit a hyperplastic growth phenotype. Increased DNA synthesis in cells from patients with DN may ultimately involve alterations in cell cycle regulatory proteins. p16 protein is a member of INK4 family of cyclin-dependent kinase inhibitors, which plays an important role in cell cycle regulation. In this study, we examined the correlation between p16 protein expression in cultured dermal fibroblasts from type 1 diabetic patients and the presence of DN.

Method. Western blot analysis was performed to compare p16 protein expression in skin fibroblasts from patients with DN as compared to control subjects, diabetic patients without DN, and nondiabetic patients with nephropathy. Transcriptional regulation of the *p16* gene was assessed using competitive reverse transcription-polymerase chain reaction (RT-PCR). Methylation status of the promoter region of p16 was examined using methylation-specific PCR, and we used single-stranded conformational polymorphism (SSCP)-PCR to assess p16 single-nucleotide polymorphism.

Results. Cells from diabetic patients with DN had nondetectable to significantly lower protein expression of p16. Similarly, mRNA expression of p16 was significantly lower in diabetic patients with DN. No hypermethylation of *p16* gene was detected, and no abnormal migrating bands were noticed on SSCP-PCR analysis in cells from patients with DN.

Conclusion. Our data indicate that cells from patients with DN exhibit significantly lower protein and mRNA expression of p16. This study could have not only important implications for the understanding of the pathogenesis of DN, but also the absence of p16 may ultimately serve as an early marker for DN.

Diabetic nephropathy (DN) remains the single most common cause of renal failure in the United States [1, 2]. The onset of proteinuria in diabetic patients is not only an indicator of renal morbidity, but is also associated with a dramatically increased risk of premature cardiovascular disease and increased mortality [3]. Approximately 30% to 40% of patients with type 1 diabetes develop nephropathy, with the incidence reaching a peak during the second decade of diabetes and sharply declining thereafter [4, 5]. This pattern suggests that only a subset of patients with type 1 diabetes is susceptible to DN, and the deleterious effects of hyperglycemia, even though necessary, are not sufficient to cause kidney disease in diabetic patients. Several lines of evidence suggest genetic susceptibility to DN [6–10]. Familial clustering of nephropathy in diabetic patients has been demonstrated [6, 7]. A diabetic sibling of a person with type 1 diabetes and nephropathy has a 72% cumulative risk of developing renal disease, whereas a diabetic sibling of a person with type 1 diabetes, but without nephropathy, only has a 25% risk [7]. To unravel the genetic basis of DN, investigators have described various cellular phenotype and genotype alterations in patients with DN [11]. We have previously shown an abnormal hyperplastic growth phenotype in cultured skin fibroblasts from type 1 diabetic patients with DN as compared to type 1 diabetic patients without DN and normal control subjects [12]. However, the underlying molecular basis for this abnormal growth phenotype is unknown.

Increasing evidence indicate that mammalian cell proliferation is ultimately regulated by the sequential activation and inactivation of a family of serine/threonine protein kinases known as cyclin-dependent kinases (CDK) [13–15]. Two families of CDK inhibitors block the activity of the cyclin-CDK complexes [13–15]. The INK4 family of CDK inhibitors ($p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$) specifically block the G1-type cyclin D/CDK4-6 complexes. The p16 locus (also known as CDKN2A) consists of two overlapping genes, each regulated by its own promoter, which through alternative reading frames encode for two unrelated proteins, p16 and $p19^{ARF}$ [16–21]. In contrast to p16, $p19^{ARF}$ is not a CDK inhibitor but

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Table 1. Clinical features of type 1 diabetic patients with and without nephropathy, nondiabetic patients with nephropathy, and control subjects

	Diabetic patients		Nondiabetics with nephropathy	Control subjects	*P* value
	With nephropathy	Without nephropathy			
Number *M/F*	8 (5/3)	8 (4/4)	6 (2/4)	8 (4/4)	—
Age *years* ± *SE*	38 ± 3	36 ± 3	41 ± 3	32 ± 2	0.165
Age range *years*	21–52	27–48	36–51	22–41	—
Duration of diabetes *years* ± *SE*	27 ± 3	25 ± 3	—		0.6814
Duration range *years*	17–47	14–37	—		—
Hemoglobin A_{1C} % ± *SE*	10.1 ± 0.4	7.8 ± 0.2	5.6 ± 0.5		<0.001
Mean arterial blood pressure *mm Hg* ± *SE*	104 ± 6	90 ± 3	99 ± 6		0.153
Albumin excretion rate *mg/24 hours* ± *SE*	2122 ± 522	7 ± 2	5017 ± 1367		<0.001
Albumin excretion range *mg/24 hours*	742–5000	3–19	2800–11700		—
Creatinine clearance *mL/min* ± *SE*	61 ± 8	123 ± 6	112 ± 17		<0.001
Creatinine clearance range *mL/min*	40–97	105–155	57–126		—

Data are shown as mean ± SE. Statistical significance is based on one-way analysis of variance (ANOVA) among different groups.

is involved in cell cycle regulation via the MDM2/p53 pathway [16]. The p16 protein binds to CDK4-6 and inhibits the activity of cyclin D/ CDK4-6 complexes [21]. Despite the widespread of alterations of p16 in senescence, immortalization, and tumorgenesis, little is known about the transcriptional regulatory mechanism of this gene. It was initially thought that p16 protein levels peak at the G_1/S transition [22]. However, further studies show that once *p16* gene expression is activated, it does not fluctuate significantly during the cell cycle [23]. It is known that the expression of p16 protein increases with age [24, 25], and interestingly, p16 protein expression is increased by the accumulation of cell doublings in culture [24, 25]. It is also well established that p16 is transcriptionally regulated [24]. Recent experimental data indicate that p16 may be inactivated by hypermethylation of its promoter region [26–30]. The *p16* gene contains a 5′CpG island in the promoter and first exon, and methylation of the cytosine base in this region has been shown to provide an important mechanism for inactivation and silencing of *p16* gene expression [26–32]. In addition to hypermethylattion, another known mechanism of p16 protein regulation is gene polymorphism. More than 150 sequence variations in the coding exons of p16 relative to the wild-type sequence have been reported [33–38]. A database of p16 mutations and polymorphism identifies at least 10 polymorphism in the coding region and 3′-untranslated region (UTR) of p16 [38].

The current study was undertaken to examine the correlation between the expression of p16 protein and the presence of DN in dermal fibroblasts from type 1 diabetic patients. In addition, we analyzed genetic and epigenetic regulation of *p16* gene in cells obtained from type 1 diabetic patients with DN.

METHODS

Patients

All procedures were approved by the Institutional Review Board at Northwestern University, and each patient gave written informed consent. Type 1 diabetic patients were recruited from the Northwestern Medical Faculty Foundation Diabetes Clinic. Type 1 diabetes was defined by the American Diabetes Association (ADA) criteria [39]. Two groups of type 1 diabetic patients, a group of age-matched normal controls subjects (eight patients in each group), and a group of nondiabetic nephropathic patients (six patients) were studied (Table 1). Diabetic patients were categorized on the basis of their urinary albumin excretion rates (AER) to either normoalbuminuric, or overt nephropathic patients. Type 1 diabetic patients excreting more than 300 mg/24-hour urine were classified as having overt nephropathy. Those patients excreting less than 30 mg/24-hour urine were classified as normoalbuminuric. Only normoalbuminuric patients with 12 or more years after the diagnosis of diabetes were included in this study. All diabetic patients with nephropathy except for one patient were taking antihypertensive medications, including angiotensin-converting enzyme (ACE) inhibitors. Nondiabetic nephropathic patients were recruited from the Northwestern Medical Faculty Foundation Renal Clinic after obtaining informed consent. Four of the nondiabetic patients with nephropathy had membranous glomerulonephritis, and two were diagnosed with focal segmental glomerulosclerosis. All of the nondiabetic patients with nephropathy were taking ACE inhibitors.

Procedures

Urinary albumin excretion was measured using a specific fluorometric immunoassay in urine samples collected over 24 hours and expressed as mg/24 hours. Glomerular filtration rate was estimated from a 24-hour measurement of the clearance of endogenous creatinine. Arterial blood pressure was measured, with a standard mercury sphygmomanometer to the nearest 2 mm Hg, in each patient after at least 10 minutes of rest in the supine position. Mean arterial blood pressure was calculated as the diastolic blood pressure plus one third of

the pulse pressure at the time of the skin biopsies. Skin fibroblasts were obtained using a standard 4 mm skin punch biopsy, as previously reported by us [40]. Briefly, biopsies were taken from the costovertebral angle of the back at an anesthetized site. Cells were dispersed with a forceps and grown in culture. Cells were fed twice weekly with Dulbecco's modified Eagle's medium (DMEM-F12) (Gibco, Grand Island, NY, USA), supplemented with 10% fetal calf serum (FCS) (U.S. Biotechnologies, Parkerford, PA, USA), penicillin (100 U/mL), streptomycin (100 μg/mL), and fungizone (250 μg/mL) at 37°C in a humidified atmosphere with 5% CO_2.

Western blot analysis

Early passaged (6 to 9 passages) skin fibroblasts were made quiescent by serum deprivation for 48 hours. For each experiment, a total of 5×10^5 cells were seeded in 150 mm^2 dishes, and at subconfluence (70% to 75% confluence), fibroblasts were made quiescent by incubation in DMEM media for 48 hours as previously described [40]. Cells were washed with phosphate-buffered saline complete (PBS), scraped off the bottom of the dish in 1 mL PBS, and microcentrifuged at 15°C. The supernatant was aspirated and the pellet was resuspended in 500 mL lysis buffer [50 mmol/L Tris-HCL (pH 7.4), 150 mmol/L NaCl, 25 mmol/L ethylenediaminetetraacetic acid (EDTA), 5 mmol/L ethyleneglycol tetraacetate (EGTA), 0.25% Na-deoxycholate, 1% Nonidet P40 (NP-40), 1 mmol/L dithiothreitol (DTT), 1 mmol/L Na metavanadate (VO3), 1 mmol/L NaF, 0.1 mg/mL phenylmethylsulfonyl fluoride (PMSF), 2 mg/mL leupeptin, 0.234 TIU/mL aprotinin, and 1 mg/mL pepstatin A]. The cells were lysed by repeated passage through a 27 gauge needle and centrifuged at 4000*g* for 15 minutes at 4°C to pellet debris, and the supernatant was collected. Following measurement of the protein concentration, 100 μg of total cellular lysate was mixed with 2 × electrophoresis sample buffer (62.5 mmol/L Tris-HCL, pH 6.7, 20% glycerol, 2% sodium dodecyl sulfate (SDS), 5% β mercaptoethanol, and 0.02% bromophenol blue), and boiled for 4 minutes. Samples were resolved in 12% SDS-polyacrylamide gel electrophoresis (PAGE), and the protein was then immunoblotted and probed with a monoclonal anti-p16 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), at a dilution of 1:500 overnight and then visualized using the ECL chemiluminescence system (Amersham Life Science, Piscataway, NJ, USA).

Competitive reverse transcription-polymerase chain reaction

To analyze *p16* gene expression in skin fibroblasts from diabetic patients with DN as compared to cells from diabetic patients without DN and in control subjects, competitive reverse transcription-polymerase chain reaction (RT-PCR) was used to quantify p16 mRNA expression by using one set of primers to amplify both the target p16 cDNA and the competitor DNA. Total RNA was extracted from cells using the Total RNA Isolation System (Invitrogen, Carlsbad, CA, USA), as described by the manufacturer. A previously prepared construct "minigene construct" was used as the template (Genbank accession No. U17140). This minigene construct contains the primer sequences for β-actin with an expected size of 224 bp. Using this construct as the template, a competitive RT-PCR product was constructed with the following primers: 5′-CAACGCACCGAATAGTTACGGACGACATG GAGAAGATCT GG-3′ (forward) and 5′-GACACGC TGGTGGTGCTGATGCGGCAGTGGCC AT-3′ (reverse). The underlined portions of the above primers are the p16 target gene primer sequences, the remaining sequences were β-actin primer sequences designed to yield a competitive product of approximately 261 bp. This product was then cloned into a plasmid vector pCR®II using the TA Cloning Kit Dual Promoter (Invitrogen) as instructed by the manufacturer. Subsequently, the ligation reaction product was transformed into TOP10F′ competent cells (Invitrogen). After isolation of plasmid DNA using the Concert Rapid Plasmid Miniprep System (Invitrogen), the plasmid DNA was digested using EcoR1 and the insert sequence was confirmed at the Northwestern University Biotechnology facility. For RT, after isolation of total RNA, first-strand cDNA synthesis was performed using the SuperScript II Reverse Transcriptase (Invitrogen) in 20 μL of the total volume. The reaction mixtures contained 50 mmol/L Tris-HCL (pH 8.3), 75 mmol/L KCL, 3 mmol/L $MgCl_2$, 10 mmol/L DTT, and 500 mmol/L each of deoxyadenosine triphosphate (dATP), ^{32}P deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP), and deoxythymidine triphosphate (dTTP). After incubation at 42°C for 2 minutes, 50 U of the SuperScript II RT and 10 U of RNase inhibitor were added and the mixture was incubated for another 50 minutes at 42°C. The reaction was terminated at 70°C for 15 minutes. Each set of amplification was carried out in 25 μL total volume containing serial dilutions of competitor, 1μL of cDNA, 10 pmol of each primer specific for the p16 or β-actin as previously mentioned, 10 mmol/L of each desoxynucleoside triphosphate (dNTP), 1 U of Taq polymerase in the standard PCR buffer with 32 cycles of 1 minute at 94°C for denaturing, 30 seconds at 58°C for annealing, and 1 minute at 72°C for extension, followed by a final extension for 10 minutes at 72°C. For p16, the two PCR products, wild-type (176 bp) and the MIMIC competitor (261 bp) were resolved on 2.0% agarose gels stained with ethidium bromide. For β-actin, the two PCR products, wild-type (461 bp) and the MIMIC competitor (224 bp) were resolved on 1.2% agarose gels stained with ethidium bromide.

Table 2. Oligonucleotide primers used for MS-PCR and polymerase chain reaction-single-stranded conformational polymorphism (PCR-SSCP) analysis

Gene/exon	Primer pairs (sense/antisense)	Size (bp)
p16-M	ttattagagggtggggcggatcgc gaccccgaccgcgaccgtaa	150
p16-U	ttattagagggtggggtggattgt caaccccaaaccacaaccataa	151
p16-exon 1	tctgcggagaggggagagcaggca gcgctacctgattccaattc	279
p16-exon 2a	acaagcttcctttccgtcatgccg ccaggcatcgcgcacgtcca	244
p16-exon 2b	ttcctggacacgctggtggt tctgagctttggaagctctcag	240
p16-exon 3	gcctgttttctttctgccctctg cgaaagcggggtgggttgt	144

Primers are shown as paired sense (top) and antisense (bottom). Abbreviations are: P16-M, p16 methylated; p16-U, p16 unmethylated.

Methylation-specific polymerase chain reaction

The CpGWIZ p16 Methylation Assay Kit was used according to manufacturer's description (Oncor, Inc., Gaithersberg, MD, USA). This assay is based on the principles of methylation-specific PCR (MSP) described by Herman et al [41]. Briefly, DNA was modified by bisulfate reaction and then underwent two separate PCR reactions using unmethylated-specific and methylated-specific amplifiers. The bisulfate treatment converts the unmethylated cytosine to uracil, while 5-methylcytosines remain unaltered. Thus, the sequence of treated DNA will differ if the DNA is originally methylated versus unmethylated. For bisulfate treatment, 1 μg of DNA was denatured by NaOH for 10 minutes at 37°C. Thirty microliters of 10 mmol/L hydroquinone and 520 μL of 3 mol/L sodium bisulfite were then added and mixed, and samples were incubated at 50°C for 16 hours. Modified DNAs were purified and eluted into 50 μL of water. Modification was completed by NaOH treatment (0.3 mol/L final concentration) for 5 minutes at room temperature, followed by ethanol precipitation. Bisulfite-modified DNA was used as a template for MSP using primers specific for either the methylated or the modified unmethylated sequences as described previously by Herman et al (Table 2) [41]. For PCR reactions, a volume of 25 μL PCR mixture containing 100 ng DNA, dNTPs at 250 μmol/L each, 1.5 mmol/L $MgCl_2$, 1 μmol/L primer, 1 × reaction buffer and 1 U of Taq polymerase (Roche Diagnostics, Indianapolis, IN, USA) was used for amplification. Reactions were performed in a 2400 Perkin-Elmer thermal cycle for 35 cycles (45 seconds at 95°C, 45 seconds at the annealing temperature of 60°C for methylated, 65°C for unmethylated PCR reaction, and 60 seconds at 72°C) followed by a 7-minute extension at 72°C. The 10 μL of PCR product was loaded onto 1.2% agarose gel and visualized by ethidium-bromide staining. A colon cancer-derived cell line (SW620) with known complete methylation of the p16 promoter was used as a positive control.

Single-stranded conformational polymorphism (SSCP)

PCR was carried out under standard conditions containing 100 ng of genomic DNA template, 2 μL 10 × PCR buffer [1× = 10 mmol/L Tris (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, and 0.01% gelatin], 3.2 μL of dNTPs (1.25 mmol/L each of dATP, dCTP, dGTP, and dTTP), 0.4 μL forward primer (20 μmol/L), 0.5 μL of reverse primer (20 μmol/L), 0.5 μL labeling reaction, 0.1 μL Amplitaq DNA polymerase (5 U/μL), and 8.3 μL dimethyl sulfoxide (DMSO) (12%). Specific set of primers for exon 1, exon 2-a, 2-b, and exon 3 are shown in Table 2. For amplification, each sample was denatured at 94°C for 5 minutes and subjected to 35 amplification cycles. Each cycle consisted of 30 seconds denaturing at 95°C, 30 seconds annealing at 65°C (for exon 3, 30 seconds at 57°C), and 30 seconds extension at 72°C, followed by 4 minutes final extension at 72°C. Subsequently, 8 μL of PCR products were denatured in 30 μL of denaturing loading dye [98% formamide, 10 mmol/L NaOH, 20 mmol/L EDTA, 0.05% (wt/vol) bromphenol blue and 0.05% (wt/vol) xylene cyanol] at 98° for 5 minutes. The samples were loaded on an 8% polyacrylamide gel and run at 1.25 vol/cm in 1× Tris-borate-EDTA (TBE) in 4°C for approximately 18 to 24 hours. After electrophoresis, the gels were fixed and stained with silver. The staining was subsequently stopped by immersing the gel in 5% acetic acid. Concurrently, following amplification of PCR products, the DNA products were sent for direct sequencing analysis at the Northwestern Sequencing Facility.

Statistical analysis

Analysis of variance (ANOVA) was used to compare the differences among different groups. The unpaired *t* test was used to test the differences between diabetic patients with and without nephropathy. *P* values ≤ 0.05 were considered significant.

RESULTS

Patient characteristics

In patients with DN, the creatinine clearance was moderately reduced as compared to diabetics without DN and nondiabetic patients with nephropathy (Table 1). By design, the mean urinary AER was significantly higher in patients with DN and nondiabetic patients with nephropathy as compared to diabetics without DN. Duration of diabetes and age were not significantly different between diabetics with and without DN. All patients with overt DN were hypertensive and were taking antihypertensive medications that included ACE inhibitors except for one patient who was not on ACE inhibitor. Glycemic control

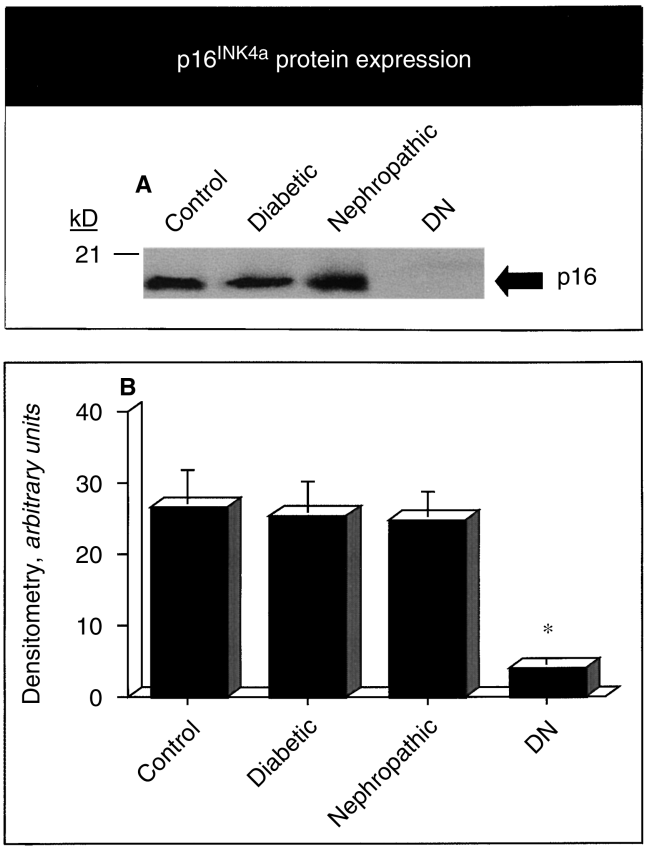

Fig. 1. $p16^{INK4a}$ protein expression. (*A*) A representative experiment of p16 protein expression in a control subject as compared to a diabetic patient without diabetic nephropathy (DN), a nondiabetic patient with nephropathy, and a diabetic patient with DN. (*B*) Relative p16 protein expression in cultured skin fibroblasts from eight control subjects, eight diabetic patients without DN, six nondiabetic patients with nephropathy, and eight diabetic patients with DN. Values are the mean densitometric values ± SE. $*P < 0.05$.

as determined by hemoglobin (HbA_{1C}) was poorer in patients with DN. Mean arterial blood pressure was not significantly different among the groups. However, diabetic patients with DN had significantly higher mean arterial blood pressure as compared to diabetics without DN.

p16 protein expression

To assess the differences in protein expression, p16 protein was measured using Western blot analysis from two separate lysates. Figure 1A shows a representative experiment. As shown in Figure 1A, p16 protein expression was undetectable or markedly lower in cells from diabetic patients with DN (4.0 ± 1.3) as compared to cells from diabetics without DN, nondiabetic patients with nephropathy, and from controls (25.3 ± 4.8 and 24.7 ± 4.1, and 26.5 ± 5.3, respectively, $P < 0.05$). Figure 1B summarizes the results of p16 protein expression indicating a significant difference in p16 protein expression among the four groups ($P < 0.05$, one-way ANOVA).

p16 mRNA expression

To examine the mRNA expression of p16 in cells from patients with DN and to confirm transcriptional regulation of p16 protein in cultured skin fibroblasts, mRNA from cells obtained from patients with and without DN in addition to control subjects were processed for competitive RT-PCR. A linearity in the ratios of PCR products of p16 cDNA in relation to plasmid DNA could be maintained when plotted against 10^{-1} to 10^{-7} serial logarithmic dilutions of the competitive plasmid DNA. Within this range of serial dilutions, the bands of *p16* gene and plasmid DNA products were discernible for densitometric analysis to obtain a ratio. In cells from patients with DN, a ratio of one was noted at 10^{-5} dilutions of the competitive DNA, while in cells from diabetic patients without DN, a ratio of one was obtained at 10^{-4} dilutions, suggesting a significant down-regulation of p16 mRNA expression in cells from patients with DN. A representative experiment is shown in Figure 2A. For the β-actin, a ratio of one was obtained at dilutions of 10^{-4} of the competitive plasmid DNA in all groups. There was no change in the β-actin mRNA expression in cells from patients with and without DN. Figure 2B shows a representative experiment. Figure 2C summarizes the result of p16 mRNA expression using competitive RT-PCR in seven diabetic patients without DN as compared to seven patients with DN. These results are consistent with data on p16 protein expression and also confirmed the altered transcription of *p16* gene in cultured skin fibroblasts from type 1 diabetic patients with DN.

Promoter methylation in the *p16* gene

The strikingly reduced levels of p16 protein and mRNA detected in fibroblasts from patients with DN led us to investigate the epigenetic mechanism of *p16* gene down-regulation by assessing the methylation status of the promoter region of the *p16* gene using methylation sensitive PCR. A colon cancer-derived cell line (SW620) with complete methylation of the p16 promoter was used as a positive control. Fibroblasts from control subjects were used as controls for the unmethylated p16 promoter. As shown in Figure 3, no aberrant methylation was detected in the *p16* gene in fibroblasts obtained from patients with DN.

PCR-SSCP analysis

The human *p16* gene structure comprises three exons, with exon 1 and 2 constituting almost 97% of the coding region of the gene [38]. Exon 1α is spliced to exons 2 and 3 to form a transcript that encodes p16, whereas exon 1β is spliced to the same exons 2 and 3 to form another novel transcript that encodes $p19^{ARF}$ in an alternative reading frame relative to p16. For mutation analysis on exon 1, 2 and 3, we carried out PCR-SSCP analysis using the primers in Table 2. Exons 1 and 3 were covered

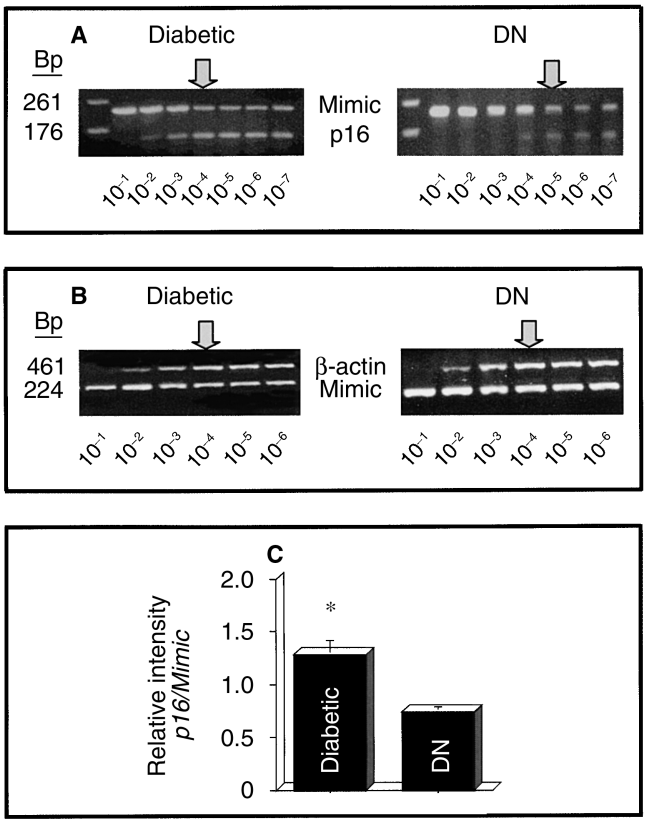

Fig. 2. Expression of p16 mRNA by competitive reverse transcription-polymerase chain reaction (RT-PCR). (*A*) A representative experiment of p16 and the competing p16 MIMIC products in a patient without diabetic nephropathy (DN) (Diabetic) as compared to a diabetic patient with DN (DN) using serial dilutions of the p16 MIMIC with a constant amount of target cDNA. The sizes for p16 and p16 MIMIC products were 176 and 261 bp, respectively. A significant decrease in the amplification of the p16 cDNA was observed in dermal fibroblasts between a diabetic patient without DN as compared to cells from a patient with DN when plotted against the log dilutions of the competitive p16 MIMIC cDNA. The arrows indicate that the ratio of one was shifted from 10^{-4} in cells from a diabetic patient to 10^{-5} in cells from a patient with DN. (*B*) For control, serial dilutions of β-actin MIMIC were amplified with fixed amount of β-actin cDNA. The sizes for β-actin and β-actin MIMIC products were 461 and 224 bp, respectively. No significant differences were observed in β-actin cDNA vs. competitive β-actin MIMIC cDNA in dermal cells from patients with and without DN. A ratio of one was confined to the 10^{-4} log dilutions of the competitive cDNA. (*C*) Expression of p16 mRNA was compared using 1×10^{-5} attomol/reaction of p16 MIMIC cDNA in seven diabetic patients with and without DN. Values are the mean densitometic values ± SE. $^*P < 0.05$.

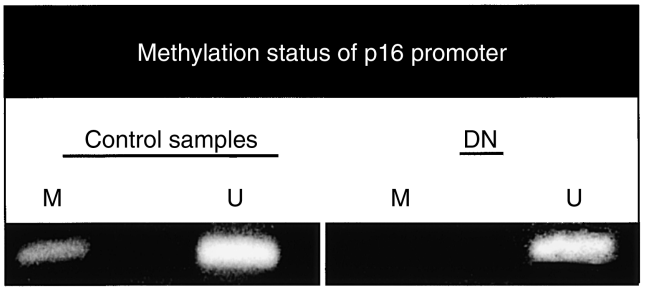

Fig. 3. Analysis of p16 promoter hypermethylation in cultured skin fibroblasts from a type 1 diabetic patient with diabetic nephropathy (DN). The polymerase chain reaction (PCR) products in lane U show the presence of unmethylated templates of *p16* gene, whereas the products in lane M indicate the presence of methylated templates. A colon cancer-derived cell line (SW620) was used as positive control. Fibroblasts from control subjects were used as control for unmethylated p16 promoter. PCR for methylated $p16^{INK4a}$ sequences shows a band in the SW620 cell line. In contrast, no amplification was observed in lane M in a patient with DN.

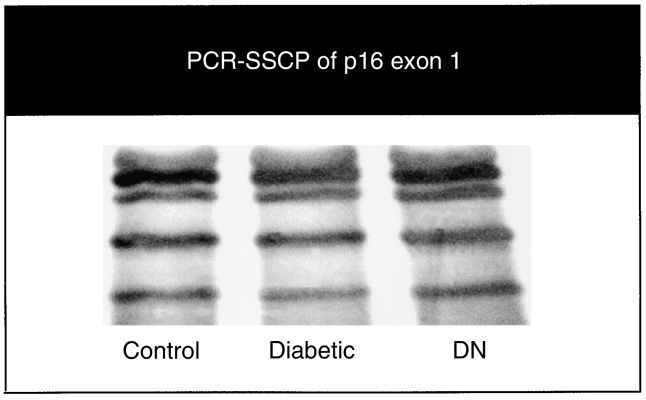

Fig. 4. Polymerase chain reaction-single-stranded conformational polymorphism (PCR-SSCP) analysis of p16 exon 1 in cultured skin fibroblasts from a control subject, a diabetic patient without diabetic nephropathy (DN), and a patient with DN. No aberrant migration fragments were noted in the sample from patient with DN. Concurrently, the DNA products were sent for direct sequence analysis of the bands shown. Direct sequencing did not show any evidence of mutations in cells from patients with DN.

with single PCR products, and exon 2 was covered by two PCR products (Table 2). PCR-SSCP analysis revealed no abnormal migrating bands in cells from patients with DN as compared to cells from diabetic patients without DN and control subjects (Fig. 4). Using DNA sequence analysis, we found no evidence of germ-line mutations within exons 1, 2, and 3.

DISCUSSION

We and others have previously shown that dermal fibroblasts from type 1 diabetic patients with DN exhibit a number of phenotypic abnormalities that include enhanced activity of the sodium-hydrogen antiporter [12, 42], enhanced collagen synthesis [43], defective antioxidant activity [44], increased α_3 integrin subunit expression [45], and an increased [^{3}H]-thymidine incorporation consistent with a hyperplastic growth phenotype [12]. All of these abnormalities suggest an intrinsically altered cell response to the diabetic milieu in cells from patients with DN. In this study, we examined the correlation between p16 protein expression in cultured dermal fibroblasts and the presence of DN. In addition, we analyzed genetic and epigenetic regulation of *p16* gene in skin fibroblasts from type 1 diabetic patients with DN.

The results of this study indicate that cultured skin

fibroblasts from type 1 diabetic patients with DN have a significantly lower protein expression of p16 as compared to cells from diabetics without DN, nondiabetic patients with nephropathy and control subjects. The difference in p16 protein expression in this study is unlikely to be due to methodologic differences. The site of the biopsy was the same in all subjects, and in each experiment, cells from diabetic patients with and without DN and controls were studied in matched sets. All the experiments were conducted under identical experimental conditions. The differences in protein expression of p16 protein is also unlikely to be due to uremia itself, as the mean glomerular filtration rate in the group of patients with DN was 61 mL/min. The effect of hyperglycemia on p16 protein expression is still largely unknown and the modulatory effect of hyperglycemia on p16 protein cannot be completely ruled out. Despite our best efforts, there was a significant difference in HbA_{1C} between the two diabetic groups (7.8 ± 0.2 mg/dL in diabetics vs. 10.1 ± 0.4 mg/dL in patients with DN). However, we could not detect any significant differences in p16 protein expression among diabetic patients without DN and nondiabetic patients with nephropathy, suggesting that a high glycemic milieu is not the only determinant of altered expression of p16 protein in cells from patients with DN.

The effect of antihypertensive therapy, especially ACE inhibitors on p16 protein, is also unknown. Recently, Wolf et al [46] showed that ACE inhibitors may modulate the protein expression of several CDK inhibitors, including p16 and p27 in vivo. The authors concluded that the use of enalapril may decrease the protein expression of p16 in BBdp rats, an autoimmune model of type 1 diabetes. In this study, the modulatory effect of ACE inhibitors, especially if it persists after several passages in vitro, on p16 protein should be considered. However, p16 protein expression was not decreased in nondiabetic patients with nephropathy despite the use of ACE inhibitors in all of those patients, suggesting that the use of ACE inhibitors is not the likely underlying mechanism of low p16 protein expression.

Little is known about the underlying regulatory mechanisms of the *p16* gene. It is well established that p16 is transcriptionally activated [38]. Several known mechanisms of transcriptional down-regulation/inactivation of the *p16* gene include hypermethylation, point mutations, small deletions, and large homozygous deletions [38]. However, somatic mutations in the *p16* gene are rare except in hereditary melanomas [37]. The initial evidence of epigenetic regulation of the *p16* gene relied on Southern blotting with methylation-sensitive restriction enzymes, particularly SacII and SmaI [26, 27, 37]. In the normal mammalian genome, methylation occurs only at cytosine (C) 5′ to guanosine (G). It seems that CpG dinucleotide-rich regions have been progressively depleted from the eukaryotic genome over evolution, except for small (0.5 to several kb) stretches of DNA, known as "CpG islands" [28, 29]. These regions are usually "protected" from methylation, and this lack of methylation might be a prerequisite for active transcription, as aberrant methylation of CpG islands has been associated with transcriptional inactivation of regulatory regions of imprinted genes. The *p16* gene contains a 5′CpG island in the promoter and first exon, and methylation of the cytosine base in this region has been shown to provide an important mechanism for silencing *p16* gene expression [26–29, 32–38]. De novo methylation of the *p16* gene has been implicated as the primary mechanism of inactivation of the gene in various cancer cell lines and in some primary tumors [26–28, 33, 38]. Our data are consistent with that of previous studies, indicating that p16 is largely transcriptionally regulated. However, we could not detect significant hypermethylation in the promoter region of the p16 in cells from patients with DN, suggesting that epigenetic regulation of $p16^{INKa}$ is not the underlying mechanism of p16 down-regulation in cells from patients with DN.

We also considered p16 polymorphism as a possible mechanism of decreased p16 expression. The *p16* gene carries several polymorphisms. The most extensively documented polymorphism of *p16* gene, A148T, results from a G>A change at nucleotide 442 which ablates a SacII site. The other two common polymorphic sites (the 500 C>G and 540 C>T) are within the 3′ UTR and the potential influences of these polymorphism on the function or expression of p16 protein is not well established [47]. It is generally accepted that frameshift mutations within exon 1 results in a truncated p16 protein causing loss of function [38]. In this study, using PCR-SSCP method for the analysis of exons 1, 2, and 3 of the *p16* gene, a uniform band migration was detected suggesting that a p16 single nucleotide polymorphism is not the underlying regulatory mechanism for the observed down-regulation of p16 expression in cells from patients with DN. Further studies are needed to unravel the regulatory mechanisms of p16 in the diabetic milieu. Currently, studies are being conducted in our laboratory to shed light on the effect of p16 and other cell cycle regulatory proteins in the hyperplastic growth phenotype in fibroblasts from patients with DN. Our results indicate that CIP/KIP family of CDK inhibitors are not altered in dermal fibroblasts in patients with DN (unpublished data).

Currently, ACE inhibitors are instituted in the course of nephropathy in patients with type 1 diabetes when microalbuminuria is detected. However, the presence of microalbuminuria may no longer be considered as an early predictor of DN, but rather an early manifestation of the disease [48]. The presence of microalbuminuria is the best available noninvasive predictor of DN risk and should be regularly measured according to established guide-

lines. However, some diabetic patients with microalbuminuria have quite advanced renal structural changes, and the presence of microalbuminuria in these cases may be a marker rather than a predictor of DN [48]. New biochemical or genetic predictors are needed to define patients who are at risk of developing DN early in the course of diabetes. If our findings are confirmed, this may have important implications. First, it may further our understanding of the pathogenesis of DN. Second, the absence of p16 may ultimately serve as an early genetic predictor of DN, allowing effective interventions such as intensive diabetes management and ACE inhibitor therapy, to be initiated very early in the course of diabetes with the resultant delay of progression or perhaps prevention of the nephropathy. Further prospective studies are needed to assess the use of p16 expression in skin fibroblasts as a predictor of DN and establish the correlation between p16 expression and genetic susceptibility to DN.

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